Exploring ubiquitin and ISG15 biology with chemical tools

Gan, J.

Chapter 2.

Highlighting the proteasome: Using Fluorescence to Visualize Proteasome Activity and Distribution

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Abstract

Proteasomes are the main proteases in the cell, responsible for the turnover of many cytoplasmic and nuclear proteins. They are essential for many cellular processes and various diseases are associated with its malfunctioning. Proteasome activity depends on the nature of the catalytic subunits, which are interchangeable, as well the interaction with associated proteasome regulators. Here we describe various fluorescence-based methods to study proteasome function, highlighting the use of activity-based proteasome probes to study proteasome localization, dynamics and activity in living cells.

Introduction

To maintain cellular homeostasis, cells must carefully balance the synthesis and degradation of cellular constituents. Proteins can fail to fold correctly during protein synthesis or can become damaged under the stress condition e.g. oxidative stress. When this happens, aberrant proteins must be swiftly removed to protect the cell from undesired protein activity and the formation of potentially toxic protein aggregates [1]. In addition, the selective and timely removal of intracellular signaling proteins by degradation at the appropriate time is important for the regulation of intracellular signaling pathways. In mammalian cells, degradation of intracellular proteins is carried out by two major pathways. The first pathway is the ubiquitin-proteasome system (UPS) and is responsible for approximately 70% of all intracellular protein degradation [2]. The second degradation pathway is autophagy which results in the lysosomal degradation of cellular components and organelles [3]. The UPS unfolds and cleaves proteins into small polypeptide fragments, with a typical length between 8-10 amino acids. These fragments can be broken down further by other proteases, providing a source of amino acids for the synthesis of new peptides. In immune cells and cells with proinflammatory cytokine stimulation, these peptide fragments can be presented by the human major histocompatibility complex-I (MHC-I) on the cell surface to the immune system [4]. Because of the important role of protein degradation in many essential cellular processes, disruption of normal proteasome function can contribute to the onset of disease [1]. The proteasome has been implicated to play a role in both neurodegenerative diseases as well as in different forms of cancer, immune-related diseases, and aging [5]. Modulation of 26S proteasome activity has proven therapeutic potential [6]. Because the regulation of 26S proteasome activity is complex, there is a high demand for assay reagents that can actually report both proteasome activity as wells as localization. This review provides an overview of the fluorescent tools that are currently available to study proteasome activity and localization. Table 1 provides an overview of the reagents discussed below.

Table 1: An overview of reagents or techniques to study proteasome activity or localization

Classes	Examples	Activity	Localization	References
Peptide-based model substrates	β1 : Z-LLE-AMC, Z-LLE-NA, Ac-nLPnLD-AMC, AC-GPLD-AMC	Yes	No	[21, 22]
	β2 : Bz-VGR-AMC, Boc-LRR-AMC, Z-ARR-AMC, Bz-FVR-AMC, Boc-LSTR-AMC, Ac-RLR-AMC	Yes	No	
	β5 : Suc-LLVY-AMC, Z-GGL-AMC, Suc-AAF-AMC	Yes	No	
FRET-based probes	Cy5-NC001, BodipyFL-NC001, Cy5-LU112, BodipyFL-LU112, Cy5-LU015, BodipyFL-LU015	Yes	No	[51, 52]
	FRET Reporter 1	Yes	No	[24]
Polyubiquitinated Model substrates	Ub5-DHFR	Yes	No	[25]
	Poly-ubiquitinated Cyclin B1	Yes	No	[26] [28]
	Poly-ubiquitinated GFP	Yes	No	[27]
	Ub4-GFP(i)	Yes	No	
GFP fusion proeins	ODC-GFP, Ub-R-GFP, Ub-L-GFP	Yes	No	[30]
Deg-On system	Deg-On, eDeg-On	Yes	No	[32]

Subunit specific ABPs*	β1c/ β1i- selective ABP β1(i) : Cy5-NC001 β2(i) : BODIPY(FL)-LU112 β5(i) : BODIPY(TMR)-NC005	Yes Yes Yes Yes	No No No No	[44] [42]
Pan-reactive ABPs*	Dansyl-Ahx3-L3-VS, BodipyFL-Ahx3-L3-VS, BodipyTMR-Ahx3-L3-VS	Yes Yes Yes	Yes Yes Yes	[33-35]
GFP	GFP-β1i		Yes	[45]

* There are comprehensive summaries about these probes available in other review articles. [41, 53]

Overview of human proteasome

The 26S proteasome is a multisubunit, ATP-dependent protease complex consisting of a 20S core particle (20S CP), capped on one or both sides by a 19S regulatory particle (19S RP) [7]. The 20S CP is composed of four stacked rings, each consisting of seven subunits. The outer two rings contain seven similar, yet distinct alpha subunits (named α1- α7). The inner two rings of the 20S CP consist of seven distinct beta subunits (named β1 - β7). Three of these beta subunits contain active sites with proteolytic activity. The constitutively expressed catalytically active subunits are β1, β2, and β5, which display caspase-like, tryptic-like, and chymotryptic-like activity, respectively. Together, these four rings form a barrel-shaped structure, with a tightly regulated gate at either end that limits the unregulated entry of substrates [7]. The regulation of this gate is controlled by a cap complex such as the 19S RP. This multisubunit complex is involved in the recognition, binding, deubiquitination, and unfolding of polyubiquitinated proteins, as well as the translocation of the polypeptide chain into the interior of the 20S CP. Alternative regulatory particles besides the 19S have been reported. These include the PA28αβ and PA28γ protein complexes, the PA200 proteasome-activating protein, and PI31 [8].

In addition to the different regulatory particles, different isoforms of the 20S CP have been described. In lymphoid tissues, or in non-lymphoid tissues after stimulation with interferon γ (IFN-γ), the constitutive β subunits can be replaced by the immunoproteasome subunits β1i, β2i, and β5i to form the immunoproteasome. Hybrid proteasomes, containing a combination of constitutive and immunoproteasomes have been reported [9], in addition

to proteasomes expressing tissue-specific subunits such as the thymoproteasome (containing β5t instead of β5) [10] and the testis-specific proteasome (containing α4s instead of α4) [11].

Proteasome activity is dynamically regulated, depending on changing cellular needs. For instance, during fundamental cellular processes such as apoptosis, proliferation, and differentiation, the activity of the proteasome is altered [5]. Environmental factors such as oxidative stress, disease states or small molecules can influence 26S proteasome activity as well [12]. One way in which proteasome activity is regulated is by post-translational modifications. Proteasomal subunits, like many other proteins, can undergo post-translational modifications such as phosphorylation [13], N-acetylation, alkylation, O-glycosylation, S-glutathionylation, N-myristoylation, and oxidation of sulfur-containing amino acid residues [14]. These modifications affect both the activity as well as the localization of the 26S complex. Another way is that the proteasome interacts with a growing list of proteasome-interaction proteins [15], including chaperones, E3 ligases and deubiquitinases which may lead to altered stability of the 26S proteasome complex and/or its proteolytic activity [16].

Additionally, proteasome activity can be regulated by small molecule compounds [17]. A wide variety of synthetic and natural inhibitors have been reported in the past 25 years, proteasome inhibitors including bortezomib and carfilzomib have been used in the clinic for the treatment of multiple myeloma and mantle cell lymphoma [18, 19]. In contrast, several drugs that increase 26S proteasome activity have potential applications in the treatment of neurodegenerative diseases [20].

Visualizing proteasome activity

1.1 Substrate-based fluorescence

The activity of both 20S and 26S proteasome can be measured using small peptide-based substrates. Such substrates are typically 3 to 4 amino acids in length and are attached to a quenched fluorescent reporter molecule such as 7-amino-4-methylcoumarin (AMC) [21] or aminoluciferin [22]. After cleavage of the substrate by the proteasome, the reporter molecule is no longer quenched, and fluorescent signal can be detected. For each of the different catalytic activities of the proteasome, there are specific peptide substrates available (Table 1). This allows the different catalytic activities of the proteasome to be measured separately. Unfortunately, many fluorogenic substrates are not cell-permeable, and therefore only applicable to study purified proteasomes, permeabilized cells, or cell lysates. Also, most substrates are processed by both the constitutive and immunoproteasome catalytic subunits of the 20S CP, and some substrates can be non-specifically processed by other proteases besides the proteasome leading to high levels of background signal. Another shortcoming of such reagents is that they do not require poly-ubiquitination or processing by the regulatory particles. However, fluorogenic substrates specific for the chymotryptic-like activity are often cell-permeable and can be used to measure proteasome activity in living cells [22]. Fluorogenic substrates also have a big advantage as they can be easily applied into high-throughput screening (HTS). For example, Suc-LLVY-AMC was used as probe to screen for small molecule agonists of purified 20S proteasome activity, and two compounds MK-866 and AM-404 were finally identified as bona fide stimulators [23].To improve upon the existing fluorescent peptide-based substrates, a peptide-based FRET reporter has been developed [24]. Compared to the classical peptide-based substrate described above, this reagent has larger size resulting

in slower degradation and increased dynamic range, is also four times higher in sensitivity and can be used in a HTS format.

1.2 Polyubiquitinated model substrates

Peptide-based model substrates differ from native poly-ubiquitinated proteasome substrates in that the latter also require recognition and processing by the proteasome regulatory particles before proteolysis. For studying the entire degradation process by the 26S proteasome, poly-ubiquitinated model substrates would be valuable research tools. Previously, the synthesis of poly-ubiquitinated model substrates such as K48 linked Ub5-DHFR [25] and poly-ubiquitinated cyclin B1 [26] have been described. Unfortunately, these model substrates are not cell-permeable and can only be used together with purified proteasomes or with cell extracts. In addition, as the readout for such reagents is gel-based, they cannot be used in high-throughput approaches. This latter limitation was overcome in a series of recent reports. One model substrate described consists of tetraubiquitin fused to GFP expressing a degradation initiation region [27]. Another GFP model substrate has K48-linked polyubiquitin chains and lacks the degradation initiation region [28]. While not cell-permeable, these reagents are highly suitable for HTS.

1.3 Intracellular model substrates

As mentioned previously, the poly-ubiquitinated model substrates reported so far are not cell-permeable. This is unfortunate, as it prevents us from studying the degradation of defined poly-ubiquitinated model substrates in its native environment. In mammalian cells it is possible to use overexpressed model substrates to determine 26S proteasome activity. One example is of such a model substrate is GFP fused to 37 amino acids of ornithine decarboxylase (ODC), a protein which is degraded in an ubiquitin-independent manner [29, 30]. Other examples of overexpressed GFP-based model substrates are fusion proteins that contain N-end rule degradation signals such as ubiquitin-R-GFP and ubiquitin-L-GFP [31]. The Deg-on system is an expression based system that translates the level of 26S proteasome activity into a fluorescent output. In this system, the expression of GFP is repressed by a continuously expressed genetically encoded proteasome substrate. When proteasome activity is increased, the level of the proteasome substrate goes down, less GFP protein expression is repressed. This results in an increased level of GFP, which can be detected. Vice versa, when proteasome activity is decreased, the levels of the proteasome substrate will rise. This will increase the repression of GFP expression, leading to lower levels of GFP[32].

1.4 Activity-based proteasome probes

Activity-based proteasome probes (ABPs) are developed based on the covalent binding of small inhibitors with active site residues of catalytic subunits. A typical ABP consists of a warhead, a recognition element and a reporter tag (Figure 1a). The recognition element, either a small polypeptide, a small molecule or a protein derivative, directs the probe to active enzyme for enhanced selectivity, then the warhead with modest reactivity covalently reacts with the catalytic residues. The reporter tag can be an affinity tag such as biotin to allow for isolation, or a fluorophore for fluorescence signal detection. The proteasome ABPs are generally classified as either subunit specific ABPs or broad spectrum ABPs based on their selectivity towards a specific or all of the catalytic subunits.

Broad spectrum ABPs are reactive to all proteasome catalytical subunits. These probes gain access to the binding target through the gated channel of the 20S core particle rather

than random diffusion. If the gate is closed, or the binding sites are occupied by a proteasome inhibitor (e.g. MG132), the fluorescence signal will decrease. Conversely, if the gate is open, more probe can enter into the 20S core particle, and the fluorescent signal will increase (Figure 1b). Dansyl-Ahx3-L3-VS was the first reported cell-permeable and directly detectable broad spectrum ABP [33]. It was subsequently optimized into two other classical proteasome probes BodipyTMR-Ahx3-L3-VS (MV151) [34] and Me4Bodipy-Ahx3-L3-VS [35], by replacing the dansyl group with Bodipy fluorophores. This change made the probe much more sensitive for fluorescence detection, while keeping its activity-based and cell-permeable properties. This means that these probes can be used in cell lysates, living cells, as well as animal tissues (Figure 1d), and is suitable for a variety of monitoring techniques, like in-gel fluorescence scan, flow cytometry and fluorescence microscopy (Figure 1c). All these features make both of these probes widely used in proteasome-related studies nowadays.

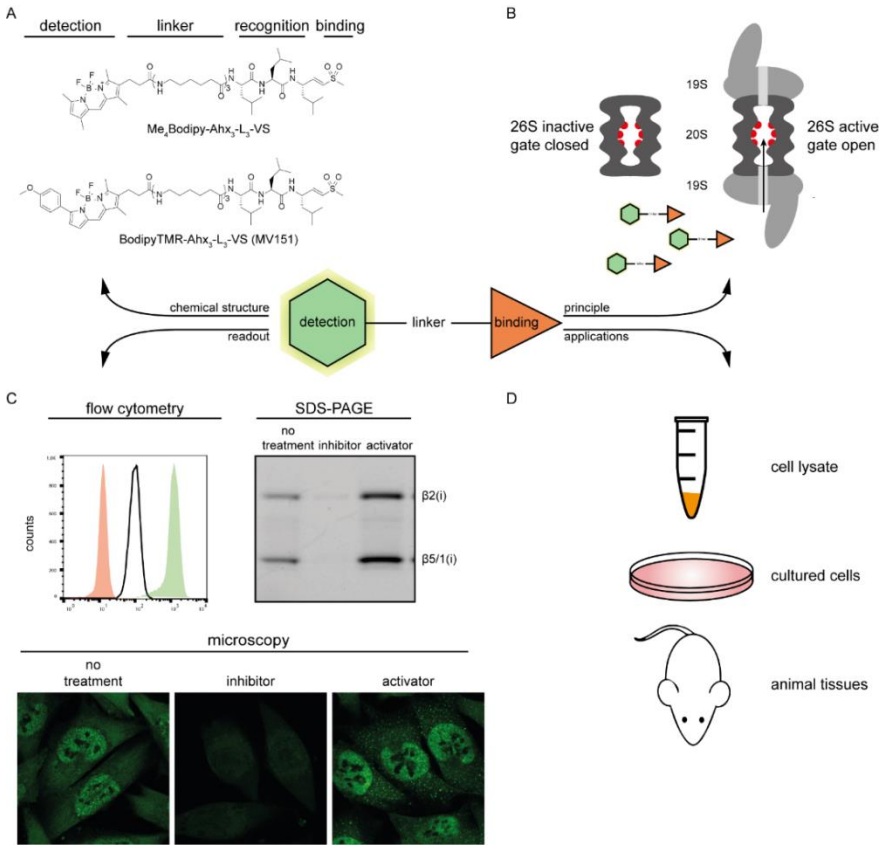

Figure 1. Overview of proteasome ABPs. A) molecular structures of 2 proteasome ABPs. B) the principle how probes target the active proteasome: proteasome ABPs enter through the 20S proteasome gate, and covalently target the catalytic sites. C) typical examples of the detection methods of proteasome ABPs. Left: overlay of the ABP signal in proteasome inhibitor treated (red), untreated (white) and proteasome activator treated (green) MelJuSo

cells. Right: In-gel fluorescence scan showing representative proteasome activity profiles of proteasome inhibitor treated, untreated and proteasome activator treated MelJuSo cells. Below: confocal microscopy images of the ABP signal in proteasome inhibitor treated, untreated and proteasome activator treated MelJuSo cells. D) application of proteasome ABPs.

The Me4Bodipy-Ahx3-L3-VS probe was used in a flow cytometry-based HTS, 11 small molecule compounds were identified as novel proteasome activators in living cells, and the p38 MAPK pathway was highlighted as a novel signal pathway to modulate proteasome activity [36]. In another research study, thermal proteome profiling revealed that CDK4/6 inhibitor palbociclib induces a thermal stabilization of the 20S proteasome complex. When MCF7 cells were treated with palbociclib, the proteasome activity also increased as measured using Me4BodipyFL-Ahx3-L3-VS probe [37]. Furthermore, the MV151 can also label the β2 and β5 subunits of Plasmodium proteasome, and was applied in a flow cytometry-based screen to identify inhibitors that selectively kill parasites [38]. Beyond applications in cultured cells, both probes are also broadly used in animal tissues, providing an easier way to study changes in proteasome activity and drug bioavailability when mice are administrated with proteasome inhibitors and activators [34, 35]. In a recent study, probing the freshly isolated neural stem cells with Me4BodipyFL-Ahx3-L3-VS probe revealed that quiescent neural stem cells (NSCs) have reduced proteasome activity compared to activated NSCs [39]. The MV151 probe has also been applied to plant science. When the living Arabidopsis plants were sprayed with benzothiadiazole (BTH), the cytoplasmic proteasome activation could be monitored by MV151 in an in-gel fluorescence scanning approach [40].

Subunit-specific ABPs have a strong preference for a specific subunit type in an optimized range of probe concentration and reaction time. A comprehensive review article about these probes was recently published [41]. For example, a cocktail of activity-based probes can be used where a combination of subunit-specific ABPs with different fluorophores can enable visualization of all six catalytic subunits simultaneously by standard SDS-PAGE gel [42]. The cocktail was then used to identfiy new subunit selective compounds, such as β5c selective inhibitors [43]. However, as these ABPs display poor cell permeability, efficient labeling requires the use of cell lysates. A cell permeable β1c/ β1i- selective ABP is available, but the fluorescence labeling requires a two-step approach [44].

Visualizing proteasome distribution and composition

1.5 Proteasome marker antibodies

The most classical approach to visualize proteasome distribution and composition is to use proteasome marker antibodies, including immunohistochemistry and immunofluorescence staining, and antibodies targeting various proteasome subunits are commercially available. This approach is broadly applicable in all kinds of cells and clinical tissues. However, this technique is quite invasive, as cell fixation and permeabilization is a premise for antibody staining, the accuracy of proteasome complex localization might be affected under the harsh treatment, like fixation with cold methanol. Besides that, the antibody cannot differentiate between active and inactive proteasomes.

1.6 Fluorescently-tagged approach

Fluorescently-tagged proteasome subunits have been widely used to visualize proteasome distribution and dynamics in living cells for over two decades, GFP-β1i was the first one

to be non-invasively incorporated into proteasome [45]. Afterwards, several other subunits were also fluorescently-tagged [46]. The intracellular distribution of fluorescently-tagged proteasomes can be easily visualized in living cells under a fluorescence microscope [47]. The dynamics of proteasomes can also be followed in time by photobleaching a small area in a living cell, if equipped with an advanced confocal microscopy with laser power, the strategy includes fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) [48]. Furthermore, the co-localization or interaction between proteasome and substrate is observable by co-expressing differently tagged proteasome subunits and substrate proteins [49]. However, application of a strategy based on fluorescently-tagged proteins is usually limited in cultured cells. Another shortcoming of this approach is that the fluorescence does not necessarily represent intracellular distribution of intact active proteasomes, because not all of the tagged subunits are efficiently incorporated in the proteasome complexes, the non-incorporated fractions can interfere with proteasome distribution, and some fluorescent pre-complexes without activity also exist in cells. Incorporation of subunits in proteasome complexes can be determined by several laborious ways: 1) proteasome complex immunoprecipitation with antibodies against different subunits; 2) sucrose density centrifugation or native gradient PAGE; 3) diffusion rate determination of the fluorescent subunits [48, 50].

1.7 ABP based approach

Proteasome ABPs target the active proteasome specifically and show a similar proteasome distribution pattern when compared to fluorescently-tagged proteasomes in living cells. The dynamics of active proteasomes in living cells can be observed when cells are treated with proteasome inhibitors, the ABP labeling is then prevented by inhibitor treatment which blocks all catalytic sites in the proteasome [35]. When mice are administrated with MV151, the profiling pictures of the proteasome in different organs or tissues can also be visualized [34].

Besides visualizing proteasome distribution, proteasome ABPs are also valuable tools in studying proteasome composition, especially subunit-specific ABPs. Two different studies described the development of a set of FRET donors and acceptors that selectively target the proteasome catalytic subunits [51, 52]. Such reagents can be used to determine the different proteasome subtypes present in cells, i.e. distinguishing constitutive proteasomes from immunoproteasomes and hybrid proteasomes. However, as these reagents are not cell-permeable, their application is limited to studying purified proteasomes, or cell lysates.

Concluding remarks

Substrate-based fluorescent reporters are useful tools for visualizing proteasome activity, but cannot be used to study active proteasome intracellular localization. While use of antibodies and fluorescently-tagged proteasome subunits are ideal approaches to visualize proteasome distribution and dynamics, they do not demonstrate proteasome activity in cells. The newly developed ABPs are a valuably bi-functional reagent to studying both proteasome activity and distribution.

Proteasome ABPs offer a series of valuable advantages over traditional assays thanks to some inherent features. First, ABPs display the availability and reactivity of the active proteasomes, rather than abundance, while antibody-based approaches detect the active and inactive forms of proteasome indiscriminately. Second, ABPs are applicable in a

natural environment, such as cell lysate or living cells, instead of being limited to assays using purified proteasome. Third, the proteasome activity can be monitored without the need to know a natural or artificial substrate of the proteasome, which remains a bottleneck for many other assays. However, there are still some limitations for proteasome ABPs, as the labeling is a covalent and irreversible reaction between the target and the probe. Labelled proteins are no longer active, and this may affect the subsequent cellular pathways in living cells. However, we are still looking forward that proteasome ABPs can play more and more important roles in proteasome related studies.

Conflict of Interest

H.O. is a founder and shareholder of Ubiq Bio B.V.

Author Contributions

JG and YL equally contributed as first author. JG, YL, and HO: Conception and design; JG and YL: manuscript writing; JG, YL, AS and HO: figure design and editing; HO: supervision. All authors approved the final version of the manuscript for publication

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